TNFα-induced cardioprotection is independent of the activation of the prosurvival kinase Erk

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INTRODUCTION

Cellular protection of the heart is the new therapeutic challenge following successes achieved with reperfusion therapy. A powerful biological phenomenon that is being scientifically exploited to dissect out cell survival programs is known as ischaemic preconditioning. Ischaemic preconditioning is evoked by sublethal transient ischaemia that paradoxically results in increased tolerance against subsequent lethal ischaemia. Identification of cell survival programs activated by preconditioning may identify new therapeutic targets for future drug development to promote cell survival in the heart.

Tumour Necrosis Factor alpha (TNFα) is a pleiotropic cytokine that has been implicated to play a role in cellular inflammation, growth, apoptosis but also cell survival. We have previously reported that ischaemic preconditioning evokes an upregulation of cardiac steady-state TNFα peptide levels in the mouse heart and a loss of protection was associated with ischaemic preconditioning in TNFα deficient mice. In addition, we have demonstrated that TNFα could mimic ischaemic preconditioning in a dose and time-dependent manner in the rat heart. However, the signalling pathways involved in the cardioprotective effect of TNFα remain poorly understood. Similar to ischaemic preconditioning, the protective effect of TNFα occurs via the sphingolipids, generation of free radicals, the activation of the signal transducer and activator of transcription-3 (STAT-3) and the phosphorylation of Bcl-2 antagonist of cell death (BAD). While ischaemic preconditioning has been shown to confer cardio-protection via the activation of the prosurvival kinase Akt, we were surprised to report that TNFα can...
protect independently of the activation of Akt\(^{(9,11)}\). The necessity for the activation of the prosurvival kinase extracellular signal-regulated kinase (Erk) during the ischaemic preconditioning stimulus has led to conflicting data in the literature\(^{(12,13)}\). However, this prosurvival kinase has never been studied during a TNF\(\alpha\) preconditioning stimulus.

Therefore, using an isolated rat heart model, the aim of this study is to investigate whether both ischaemic preconditioning- and TNF\(\alpha\)-induced cardio-protection require the activation of Erk during the preconditioning stimulus also called the trigger phase.

**METHODS**

**Animal groups**

All the experiments were conducted on adult male Long-Evans weighing 250-300g in accordance with the Guide for the Care and Use of Laboratory Animals (National Academic Press, Washington DC, 1996). The University of Cape Town Medical School Ethics Committee approved all experiments.

**Experimental protocols**

Rats were anesthetised with 60mg/kg intraperitoneal sodium pentobarbitone and were given an intravenous injection of 200IU heparin. Hearts were excised rapidly and perfused retrogradely using the Langendorff technique at a constant pressure (100 cm H\(_2\)O) with oxygenated Krebs-Henseleit buffer as previously described\(^{(6)}\). A balloon was inserted through the left atrium into the left ventricle and the left-ventricular end diastolic pressure (LVEDP) was adjusted between 4 and 8mmHg. Cardiac parameters were monitored continuously and included heart rate (HR), left ventricular developed pressure (LVPD, difference between left ventricular end-systolic pressure and end-diastolic pressure) and the coronary flow (CF).

The perfusion protocol is shown in Figure 1. All hearts were allowed to equilibrate for 15 min and were consequently subjected to a standard 30 min regional ischaemia (induced by tightening a snare around the left coronary artery) followed by 120 min of reperfusion. Ischaemic preconditioning (IPC) was performed by 2 cycles of 5 min global ischaemia-reperfusion prior to the standard ischaemia. TNF\(\alpha\) (0.5ng/ml) was given for 7 min followed by a 10 min washout period before the standard ischaemia. Additional groups were perfused with the MAPK/Erk 1/2 kinase (MEK 1/2) inhibitor PD 98059 (10μM) for 15 min followed by 5 min washout prior to the ischaemia-reperfusion insult.

For infarct size measurement, the coronary artery was reoccluded at the end of the reperfusion period and a solution of 2.5% Evans Blue was perfused to delineate the area at risk. Hearts were then frozen and cut into slices, incubated in sodium phosphate buffer containing 1% w/v triphenyltetrazolium chloride (TTC) for 15 min to visualise the unstained infarcted region. Infarct and risk zone areas were determined with planimetry and infarct was expressed as a percentage of the risk zone.

**Western blot analysis**

Hearts from control or preconditioned rat hearts were collected prior to the 30 min of regional ischaemia. The ventricular tissue was excised and freeze-clamped in liquid nitrogen before being stored at -80°C. Cytosolic proteins were extracted as previously published\(^{(9)}\). Phosphorylated proteins (phospho-Erk, thr 202/thr 204), total levels of Erk and β-actin were analysed by SDS-PAGE immune-electrophoresis using antibodies from Cell Signalling Technology. Equal loading was verified with Ponceau staining or with β-actin and levels of phosphorylated proteins were normalised to their total protein levels done in the same samples and in the same conditions but on a separate
membrane. Relative densitometry was determined using a computerised software package.

**Pharmacologic agents**
Recombinant rat tumour necrosis factor alpha was obtained from Pepro Tech EC Ltd (London, UK). All the chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA).

**Statistical analysis**
Data are presented as mean ± SEM (n≥6). Comparisons between multiple groups were performed by one-way ANOVA followed by the Student-Newman-Keul post hoc test (Graph Pad Instat). A value of p<0.05 was considered statistically significant.

**RESULTS**

**PD 98059 does not influence the infarct size in ischaemic or TNFα preconditioning.**

To investigate the role of Erk 1/2, we measured infarct size as the percentage of the area at risk in hearts perfused with an inhibitor of MEK 1/2 (PD 98059). Ischaemic control hearts had an area at risk of 46.4±1.5% and an infarct size of 28.4±3.7% (Figure 2). The size of the infarct confirms previous data from our laboratory.\(^{6,9,14}\) Although the area at risk did not differ amongst the different groups (data not shown), both IPC and pharmacological preconditioning with TNFα reduced the infarct size compared with the ischaemic control group (3.2±1.6 and 6.6±2.0% respectively; p<0.001 vs. ischaemic control group). When PD98059 was given during the preconditioning stimulus, the protective effects of neither IPC nor TNFα were abrogated (3.9±1.4 and 10.6±3.4% respectively; p<0.001 vs. ischaemic control group). Of note, PD98059, given on its own, did not affect the infarct size compared with the ischaemic control group (30.5±4.0%, ns vs. ischaemic control group).

**IPC but not TNFα activates phosphorylation of Erk 1/2 during the preconditioning stimulus.**

We explored whether Erk was phosphorylated following the preconditioning stimulus with ischaemia or with TNFα. As shown in Figure 3, IPC significantly phosphorylated cytosolic Erk 1/2 compared with the control group (8.1±0.4 arbitrary units (AU) for IPC versus 5.1±0.5 for the control group, p<0.01). In contrast, TNFα preconditioning stimulus did not affect phosphorylation of Erk 1/2 (3.7±0.5 AU).

**FIGURE 2:** Effect of PD98059 (10μM) on the resistance to myocardial infarction conferred by ischaemic preconditioning (IPC) and TNFα (0.5ng/ml). Infarct size was measured following 30min occlusion of the left coronary artery and 120 min of reperfusion.

* p<0.05 versus control ischaemia/reperfusion group (CTL I/R).

**FIGURE 3:** Representative Western blots demonstrating cytosolic levels of phosphorylation of Erk following the preconditioning stimulus in isolated rat hearts. Densities expressed in arbitrary units (AU) show that ischaemic preconditioning (IPC) but not TNFα phosphorylates Erk.

* p<0.05 versus control group (CTL).
DISCUSSION

Using the isolated rat heart model, our novel data show - for the first time - that TNFα mimics ischaemic preconditioning independently of the activation of the prosurvival kinase Erk 1/2 prior to the ischaemic insult. In addition, we report that ischaemic preconditioning is associated with an increased phosphorylation of Erk 1/2 prior to the index ischaemia but its inhibition does not abrogate the protection afforded by ischaemic preconditioning.

Previous studies have explored the role of Erk in ischaemic preconditioning but the results have led to conflicting data. Using an in vivo model of ischaemia/reperfusion in rats, Fryer et al. reported an increased phosphorylation of Erk following a preconditioning stimulus with either ischaemia or an opioid receptor agonist. In addition, inhibition with PD 98059 abolished the cardio-protective effect of both preconditioning stimuli. Similarly, inhibition of Erk with PD 98059 could abolish the ischaemic preconditioning effect in the porcine heart in vivo. In contrast, Behrends et al. failed to find any correlation between Erk phosphorylation and the protection afforded by ischaemic preconditioning. In the isolated rat heart model, Mocanu et al. demonstrated that Erk phosphorylation occurred as a result of ischaemic preconditioning but was not required for protection. Using different doses of PD 98059 to inhibit the phosphorylation of Erk they also confirm that their findings were not dependent on the concentration of the inhibitor used in their model. Using the same model with a different strain of rats, our data correlated with Mocanu’s findings, thus suggesting that the discrepancies described in the literature may be related to the different species and models used.

In ischaemic preconditioning and with numerous pharmacological agents, the phosphorylation of this kinase is required at the onset of reperfusion as part of the activation of the Reperfusion Injury Salvage Kinase pathway (RISK). However, we were surprised to report that pharmacological preconditioning with TNFα protects independently of the phosphorylation of Erk at the time of reperfusion. In addition, its inhibition during the reperfusion phase did not alter the protection afforded by the TNFα stimulus. If we had previously shown that Erk is not a mediator in TNFα-induced preconditioning our present data strongly suggest that Erk does not act as a trigger either. In cardiomyocytes, previous reports have described a phosphorylation of Erk following TNFα exposure but the cells were exposed to higher and harmful doses of TNFα. In addition, the activation of Erk in the cardiomyocytes exposed to 10ng/ml of TNFα (dose 20 times higher than the dose used in our model) was so low that it was undetectable with a classic Western blot technique. It is appropriate to suggest that a very small phosphorylation of Erk may have occurred in our model but its inhibition with PD 98059 did not alter the cardio-protective effect of TNFα, therefore suggesting that Erk is not required for TNFα to promote cardio-protection. However, it is important to acknowledge that the use of non specific pharmacological inhibitors such as PD 98059 is a limitation to our study. Unfortunately, there is no inhibitor specifically targeting the kinase Erk and further studies, using either genetically modified mice such as Erk null mice or siRNA techniques, should be considered to confirm our findings.

The signalling cascade leading to cardio-protection with TNFα still remains poorly understood. Tanno et al. reported that TNFα-induced protection of the murine heart is independent of p38-MAPK activation. We have shown that the activation of sphingolipids, protein kinase C, the mitochondrial potassium ATP-dependent and reactive oxygen species were required during the preconditioning phase for TNFα to confer cardio-protection. The mitochondrial permeability transition pore and the calcium-activated potassium channel have also been suggested. Most importantly, we have recently demonstrated that the activation of STAT-3, as part of the Survivor Activating Factor Enhancement (SAFE) pathway, is a novel and alternative protective pathway that is activated during the trigger phase in both ischaemic preconditioning and pharmacological preconditioning with TNFα.

In conclusion, our present data demonstrate that both ischaemic preconditioning and TNFα can confer protection against ischaemia-reperfusion, independently of the activation of the prosurvival kinase Erk prior to ischaemia. Combined with our already published work, these data add proof that multiple and independent protective paths can be activated within the heart and that these pathways do not need to be all activated to protect the cells from dying. However, additional work is needed to understand...
whether activation of several pathways at the same time can confer an additive effect to maximise the protection or if the threshold of protection is already achieved by activation of a single path.

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